Retinal Pigment Epithelium Lipofuscin Proteomics*

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Lipofuscin accumulates with age in the retinal pigment epithelium (RPE) in discrete granular organelles and may contribute to age-related macular degeneration. Because previous studies suggest that lipofuscin contains protein that may impact pathogenic mechanisms, we pursued proteomics analysis of lipofuscin. The composition of RPE lipofuscin and its mechanisms of pathogenesis are poorly understood in part because of the heterogeneity of isolated preparations. We purified RPE lipofuscin granules by treatment with proteinase K or SDS and showed by light, confocal, and transmission electron microscopy that the purified granules are free of extragranular material and associated membranes. Crude and purified lipofuscin preparations were quantitatively compared by (i) LC MS/MS proteomics analyses, (ii) immunoanalyses of oxidative protein modifications, (iii) amino acid analysis, (iv) HPLC of bisretinoids, and (v) assaying phototoxicity to RPE cells. From crude lipofuscin preparations 186 proteins were identified, many of which appeared to be modified. In contrast, very little protein (~2% (w/w) by amino acid analysis) and no identifiable protein were found in the purified granules, which retained full phototoxicity to cultured RPE cells. Our analyses showed that granules in purified and crude lipofuscin preparations exhibit no statistically significant differences in diameter or circularity or in the content of the bisretinoids A2E, isoA2E, and all-*trans*-retinal dimer-phosphatidylethanolamine. The finding that the purified granules contain minimal protein yet retain phototoxic activity suggests that RPE lipofuscin pathogenesis is largely independent of associated protein. The purified granules also exhibited oxidative protein modifications, including nitrotyrosine generated from reactive nitrogen oxide species and carboxyethylpyrrole and iso[4]levuglandin E_2 adducts generated from reactive lipid fragments. This finding is consistent with previous studies demonstrating RPE lipofuscin to be a potent generator of reactive oxygen species and supports the hypothesis that such species, including reactive fragments from lipids and retinoids, contribute to the mechanisms of RPE lipofuscin pathogenesis. ***Molecular & Cellular Proteomics 7:1397–1405, 2008.***

Lipofuscin is a heterogeneous, fluorescent waste material that accumulates with age in active postmitotic cells such as cardiac myocytes, select neurons, and the retinal pigment epithelium (RPE)[1] (for reviews, see Refs.1–4). RPE lipofuscin, monitored *in vivo* as fundus autofluorescence, may be associated with retinal degenerative diseases such as Best macular dystrophy, Stargardt disease, and age-related macular degeneration (AMD) (1, 4, 5). Although studied intensely for over 30 years, the molecular composition and pathogenic mechanisms of RPE lipofuscin remain poorly defined. This is due in large part to the heterogeneous nature of most isolated lipofuscin preparations. RPE lipofuscin granules are considered membrane-bound residual bodies of the lysosomal compartment of the cell (1, 4). Estimates suggest that lipofuscin contains 30–70% protein depending upon tissue, species, and study (2, 6) and that this protein may contribute to pathogenesis. From 2002 to 2007 proteomics investigations have reported protein compositions for RPE lipofuscin (6, 7) and RPE melanolipofuscin (8, 9). Oxidative protein modifications have also been associated with lipofuscin (6, 7, 10), but studies to date have not demonstrated that the identified proteins and oxidative modifications are from the granules rather than from the contaminating material that co-purifies with the gran-

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[1] The abbreviations used are: RPE, retinal pigment epithelium; AMD, age-related macular degeneration; CEP, 2-(ω-carboxyethyl)pyrrole; A2E, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*,7*E*-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6,-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*-hexatrienyl]-pyridinium; mAb, monoclonal antibody; NEM, *N*-ethyl morpholine acetate; iso[4]LGE_2, iso[4]levuglandin E_2; TEM, transmission electron microscopy; DAB, diaminobenzidine; DHA, docosahexaenoyl.

ules. Because *in vitro* bioactivity studies of lipofuscin have also utilized preparations that contain extragranular contaminants, a clear understanding of the source of the bioactivities demonstrated in these studies remains elusive.

In the RPE, lipofuscin is derived primarily from phagocytosis of shed photoreceptor outer segments and is associated with a functioning retinoid visual cycle. For example, RPE lipofuscin is significantly reduced in mice lacking the isomerohydrolase (RPE65) that generates 11-*cis*-retinol (11) and also by small molecules that disrupt the visual cycle or that reduce serum vitamin A (12–15). Numerous fluorophores have been detected in RPE lipofuscin such as the pyridinium bisretinoid isomers A2E and isoA2E (16–18), oxidized derivatives of A2E, and conjugates such as all-*trans*-retinal dimer-phosphatidylethanolamine (4, 19). Several *in vitro* studies have shown that exposure of RPE cells to short wavelength light (390–550 nm) after engulfing lipofuscin or A2E results in cell death (20–23). The phototoxicity of A2E to RPE cells is well established; however, recent *in vitro* studies have also associated complement activation (24) with A2E in RPE cells exposed to light and shown that A2E perturbs cholesterol metabolism in RPE cells without light exposure (25). Such bioactivities support a possible role for lipofuscin in AMD progression, but the molecular mechanisms remain to be determined.

Toward a better understanding of lipofuscin pathogenic mechanisms, we initiated studies to define the RPE lipofuscin proteome. However, our early efforts were complicated by the significant heterogeneity of lipofuscin preparations. Sample preparation is perhaps the most important part of proteomics analysis; therefore we purified lipofuscin granules free of extragranular debris. The purified granules were analyzed on a quantitative basis for proteins, amino acids, oxidative modifications, bisretinoids, and phototoxicity. Our results show that the highly purified lipofuscin organelles contain oxidative modifications and bisretinoids and are phototoxic to RPE cells but contain only a minimal amount of protein.

EXPERIMENTAL PROCEDURES

RPE Lipofuscin Preparations—Human eyes were obtained from the Bristol Eye Bank, Bristol, UK with permission for research in accordance to local ethical requirements. RPE cells were isolated from human eyecups by gentle brushing in PBS and stored at −80 °C. Lipofuscin was isolated from RPE lysates using high speed ultracentrifugation in a discontinuous sucrose density gradient (2.0–0.3 M) as described previously (26) and designated "crude" lipofuscin. Three crude lipofuscin preparations were analyzed: preparation 1, from 65 donors (130 eyes), mean age 70 years; preparation 2, from 30 donors (60 eyes), mean age 65 years; and preparation 3, from 41 donors (82 eyes), mean age 76 years. Crude lipofuscin was further purified by either (i) washing six times in 15 mM *N*-ethyl morpholine acetate (NEM), pH 8.3, 2 mM EDTA, 100 μM butylated hydroxytoluene, 0.2% SDS or (ii) digestion with proteinase K (10 μg/μl; 24 h at room temperature) in 15 mM NEM, pH 8.3, 2 mM EDTA, 100 μM butylated hydroxytoluene, 0.2% SDS. Aliquots of crude lipofuscin, SDS-washed lipofuscin, and proteinase K-digested lipofuscin were quantified by dry weight with a Sartorius Model SE2 microbalance (Data Weighing Systems, Inc.). Granules were also quantified by counting with a hemocytometer and microscope.

SDS-PAGE and Western Analysis—For Western analysis, lipofuscin preparations were extracted with chloroform/methanol (2:1, v/v), and the chloroform-insoluble material was sonicated and then boiled in SDS sample buffer containing 0.1 M DTT. Chloroform-insoluble lipofuscin material (~20 μg) was applied to PVDF membrane (Millipore) using a slot blot apparatus (Invitrogen HS-1878) and probed with one of the following anti-adduct antibodies: mouse anti-nitrotyrosine mAb (Millipore), mouse anti-2-(ω-carboxyethyl)pyrrole (CEP) mAb, and rabbit polyclonal anti-iso[4]levuglandin E_2 (iso[4]LGE_2) (27). Alternatively the chloroform-insoluble material was fractionated by SDS-PAGE, blotted to PVDF membrane, and probed with anti-CEP polyclonal antibody as described previously (27).

Protein Identification by LC MS/MS—Crude and proteinase K-treated lipofuscin (~170 μg dry weight each) were sonicated, then boiled for 5 min in Laemmli SDS-PAGE sample buffer, subjected to SDS-PAGE on 10% gels, and stained with colloidal Coomassie Blue (Gel Code Blue, Pierce). Gel slices were excised from the top to the bottom of the lane; proteins were reduced with DTT (10 mM), alkylated with iodoacetamide (55 mM), and digested *in situ* with trypsin; and then peptides were extracted for LC MS/MS (27). LC MS/MS was performed with a QTOF2 instrument (Waters) using a Cap LC system (Waters), a 0.3 × 5-mm trapping column (C_{18} PepMap 100, LC Packings), a reverse phase separating column (75 μm × 5 cm, Vydac C_{18}), and a flow rate of 250 nl/min (27). Gradient LC separation was achieved with aqueous formic acid/acetonitrile solvents. The QTOF2 mass spectrometer was operated in standard MS/MS switching mode with the three most intense ions in each survey scan subjected to MS/MS analysis.

Alternatively equal amounts (by dry weight) of crude or SDS-washed lipofuscin preparations were subjected to overnight tryptic digestion in 30 mM NEM, pH 8.6, 0.05% SDS containing 0.3 μg of trypsin, and soluble components were fractionated by strong cation exchange chromatography using a PolySULFOETHYL Aspartamide column (1.0 × 150 mm, 5-μm particle size, 200-Å pore size), a flow rate of 50 μl/min, and a gradient of 0–600 mM KCl in 25% acetonitrile, 10 mM KH_2PO_4, pH 3, with fractions collected at 1-min intervals. Strong cation exchange fractions were analyzed by LC MS/MS on a QTOF2 mass spectrometer as described above.

Protein identification from QTOF2 MS data utilized MassLynx 4.1 software (Waters), the Mascot search engine (Matrix Science, version 2.1), and the Swiss Protein Sequence Database (August 21, 2007, version 54.1). The Swiss Protein Database search parameters included all human entries (~17,000 total sequences), one missed tryptic cleavage site allowed, a precursor ion mass tolerance of 0.8 Da, a fragment ion mass tolerance of 0.8 Da, and protein modifications for Met oxidation and Cys carboxyamidomethylation. A minimum Mascot ion score of 25 was used for accepting all peptide MS/MS spectra. A minimum of two unique peptides per protein was required for all protein identifications.

Amino Acid Analysis—Aliquots of lipofuscin preparations of known dry weight were subjected to vapor phase HCl hydrolysis (150 °C for 1 h), and phenylthiocarbamyl amino acid analysis was performed as described previously (28) using an Agilent 1100 HPLC system equipped with an autosampler, a Gilson 116 UV detector, and an Applied Biosystems 112A column oven.

Light, Transmission Electron, and Confocal Fluorescence Microscopy—Both crude and purified lipofuscin granules were fixed in 2% glutaraldehyde and 1% paraformaldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol to propylene oxide, embedded in Epon/Araldite, and polymerized for 48 h at 60 °C (27). For light microscopy, semi-thin sections were cut with a diamond histotech knife, dried, and strained with toluidine blue. Slides were

photographed with a Zeiss Axiophot microscope equipped with a Hamamatsu digital camera. For transmission electron microscopy (TEM), the same block of plastic-embedded samples was thin sectioned on an RMC MT-XL ultramicrotome, stained with uranyl acetate and lead citrate, and viewed in a Tecnai 20, 200-kV digital electron microscope equipped with a Gatan image filter. For immuno-TEM, crude lipofuscin or purified granules ($\sim 10^8$ granules) were incubated in PBS containing 2.5 μg of anti-CEP mAb and 1% BSA for 1 h at room temperature. The resulting pellet was washed three times with ice-cold PBS containing 1% BSA and 0.1% Triton X-100 and then incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG for 1 h at room temperature. After three additional washes, diaminobenzidine (DAB) was added to 0.5 mg/ml and incubated until brown color formation ($\sim$20 min) after which the pellet was washed two times and processed for TEM as described above. Negative controls lacked primary antibody. For confocal microscopy, crude and purified lipofuscin granules were analyzed with a Leica laser scanning confocal microscope (TCS-SP2) using a high magnification, oil immersion objective lens. Images were collected sequentially in the green, red, and differential interference contrast channels through the entire sample using a 1 μm step size.

Morphometric Analyses—Morphometric indices of lipofuscin granules including area, number, roundness, aspect ratio, perimeter, and mean diameter were extracted from TEM images using a semiautomated batch-processing routine generated in Image-Pro Plus 6.2 (Media Cybernetics). Roundness (also considered a measure of smoothness) was determined by the formula $(\text{perimeter})^2/(4\cdot\pi\cdot\text{area})$. In this measurement, roundness = 1 for circular objects, whereas roundness is greater than 1 for other shapes. Aspect ratio concerns the circularity of the granule, is always $\geq$1, and was determined by major axis/minor axis. Roundness and aspect ratio are ratios and do not have units.

HPLC Analysis of Bisretinoids—Crude and SDS-washed lipofuscin samples were extracted with chloroform/methanol (2:1), filtered first through cotton and then through a C_{18} Sep-Pak cartridge (Millipore) in methanol containing 0.1% TFA, and dried under argon. The redissolved extract was analyzed on an Alliance HPLC system (Waters) equipped with a 2695 Separation Module, a 2996 Photodiode Array Detector (with monitoring at 430 and 510 nm), Empower® software, and an Atlantis® dC_{18} column (3 μm, 4.6 $\times$ 150 mm, Waters) using aqueous trifluoroacetic acid/acetonitrile gradients with a flow rate of 0.8 ml/min as described previously (18). The bisretinoid lipofuscin compounds A2E, isoA2E, and all-*trans*-retinal dimer-phosphatidylethanolamine were identified and quantified based on UV-visible absorbance spectra and elution times that correspond to authentic synthetic compounds (4).

Phototoxicity Assay—Confluent ARPE-19 cells were fed equal amounts (by granule count) of crude or SDS-washed lipofuscin and then maintained in basal medium as described previously (21). Control cells lacked lipofuscin. After 7 days the basal medium was replaced with photosensitizer-free medium (SF10PF), and cells were maintained in the dark or exposed to blue light (400–500 nm) at 2.8 milliwatts/cm^2 at 35 °C for 48 h. Cell viability was then assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (21).

RESULTS

Purification of Lipofuscin Granules—RPE lipofuscin granules are usually isolated by sucrose density gradient centrifugation (26) and typically contain significant amounts of extragranular debris as shown (Fig. 1, *A*, *C*, *E*, *F*, *I*, and *K*) by light, confocal, and transmission electron microscopy. In the crude preparations the lipofuscin granules showed extensive clumping, and distinct aggregate domains were evident in the pellet (Fig. 1*A*). In wet preparations viewed with microscopy large extragranular debris were evident that were not associated with lipofuscin fluorescence (Fig. 1, *E* and *F*). We used two different approaches to remove the extragranular debris prior to structural and functional characterization of the granules. Treatment of crude lipofuscin preparations with proteinase K effectively removed the extragranular material without changing the gross structure of the granules (Fig. 1, *G*, *H*, and *J*). Subsequently we found that simply washing crude lipofuscin preparations with the digestion buffer containing 0.2% SDS but no proteinase K also removed the extragranular debris (Fig. 1, *B*, *D*, and *L*). High magnification TEM analyses revealed that most granules in the crude preparation were surrounded by membranous material, whereas purified granules lacked membranes (supplemental Fig. 1). However, as shown in supplemental Fig. 2, morphometric analyses of 472 granules in a crude lipofuscin preparation and 1711 purified granules revealed no statistically significant difference in granule diameter (crude, 0.74 $\pm$ 0.24 μm; purified, 0.76 $\pm$ 0.20 μm; $p = 0.12$) or circularity (aspect ratio: crude, 1.23 $\pm$ 0.81; purified, 1.25 $\pm$ 0.18; $p = 0.06$). Measurements for roundness (smoothness) suggested possible differences between granules from purified and crude preparations (crude, 1.06 $\pm$ 0.09; purified, 1.08 $\pm$ 0.09); however, we do not consider these differences to be significant. The roundness metric utilized an algorithm that separates two or more touching granules generating virtual corners with the consequence that "roundness" values would appear less smooth for the purified granules because of closer packing and more granules than in the crude preparation image.

Lipofuscin Proteomics—Proteomics analyses of three lipofuscin preparations were pursued. Equal amounts (by dry weight) of crude and proteinase K-treated lipofuscin were fractionated by SDS-PAGE, gel slices were excised, *in situ* tryptic digestion was performed, and proteomics analyses were pursued by LC MS/MS. From crude lipofuscin preparation 1, 77 proteins were identified (supplemental Table 1); however, no proteins were identified from proteinase K-treated lipofuscin except two background proteins (trypsin and keratin). Many of the proteins in crude lipofuscin were detected in multiple gel slices and at significantly higher apparent mass than predicted, suggesting the presence of heavily modified and possibly cross-linked proteins (supplemental Table 1). Proteomics analysis of the chloroform-insoluble fraction of crude lipofuscin preparation 2 following SDS-PAGE fractionation yielded similar results (124 proteins identified), supporting the presence of modified proteins (supplemental Table 2). For proteomics analyses of lipofuscin preparation 3, equal amounts (by dry weight) of crude or SDS-washed lipofuscin were digested in a dilute detergent solution, peptides were fractionated by strong cation exchange chromatography, and subsequent LC MS/MS yielded the identity of 75 proteins (supplemental

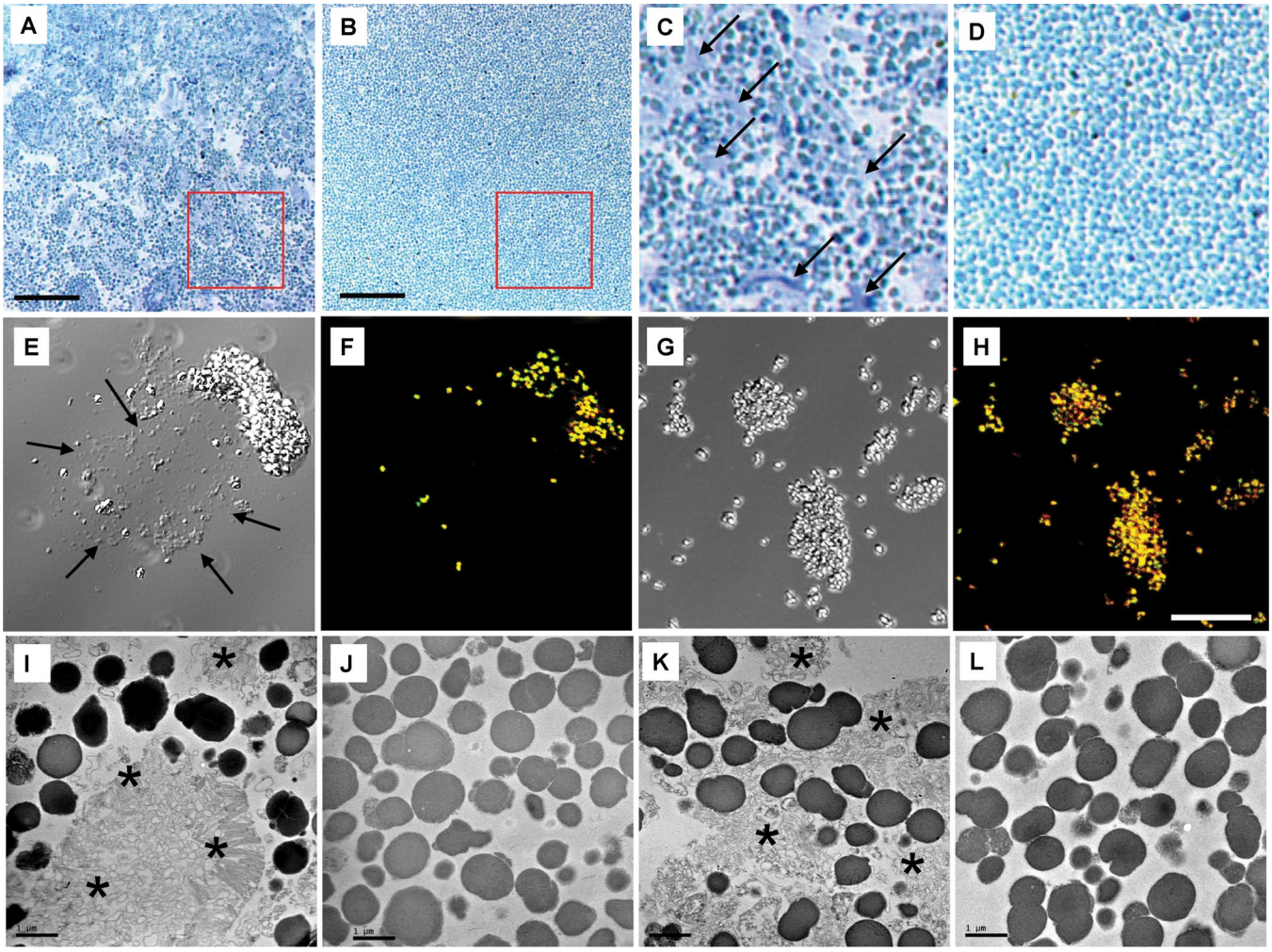

FIG. 1. **Light, fluorescence confocal, and transmission electron microscopy of lipofuscin.** Light microscopy of crude lipofuscin (*A*) and SDS-washed lipofuscin granules (*B*) stained with toluidine blue. *Scale bar*, 20 μm in *A* and *B*. Note the clumped, non-homogeneous appearance of the material in the crude preparation in *A*, whereas in *B* the lipofuscin granules show homogeneous packing. Higher magnification of the *boxed area* indicated in *A* is presented in *C*. Note the extensive lightly staining extragranular material present in *C*, some of which is indicated with *arrows*. The *boxed area* indicated in *B* is presented at higher magnification in *D*. Note the distinct staining of the individual granules and the absence of any extragranular material in this SDS-washed preparation. Differential interference contrast images (*E* and *G*) and corresponding laser-excited autofluorescence (*F* and *H*) microscopy of wet preparations of lipofuscin isolates from crude lipofuscin (*E* and *F*) and proteinase K-treated lipofuscin granules (*G* and *H*) are shown. In the crude preparation in *E* an extensive array of extragranular debris is indicated by *arrows* that does not correspond to areas of lipofuscin granule fluorescence in *F*. This extragranular material is not evident in *G*. Images in *E–H* are presented at identical magnifications. *Scale bar*, 16 μm in *H*. Transmission electron micrographs of crude lipofuscin (*I* and *K*), lipofuscin granules after proteinase K treatment (*J*), and SDS-washed lipofuscin granules (*L*) are shown. Significant extragranular debris are present in crude lipofuscin (some indicated by *asterisks*) but are absent in the SDS-washed or proteinase K-treated lipofuscin granules.

Table 2). A total of 186 proteins were identified from the three crude lipofuscin preparations, but no proteins were identified from the purified granules. LC MS/MS analysis of tryptic digests from the purified granules yielded total ion current chromatograms that exhibited a few unidentifiable peaks that were of very low intensity relative to peptides from trypsin and keratin.

Oxidative Protein Modifications in RPE Lipofuscin—Crude lipofuscin and SDS-washed lipofuscin granules were probed by Western slot blot analysis for nitrotyrosine and modifications derived from oxidative fragmentation of docosahexaenoyl (DHA)- or arachidonyl-containing lipids. Both crude lipofuscin and the purified granules exhibited immunoreactivity to CEP adducts from DHA (27), to iso[4]LGE_2 adducts from arachidonyl-containing lipids (29), and to nitrotyrosine (Fig. 2, *A*, *B*, and *C*). Western analysis following slot blot was used because SDS-PAGE of the purified granules failed to yield either Coomassie Blue-stained or immunoreactive components (not shown). However, analysis of crude lipofuscin following SDS-PAGE demonstrated Coomassie Blue bands and

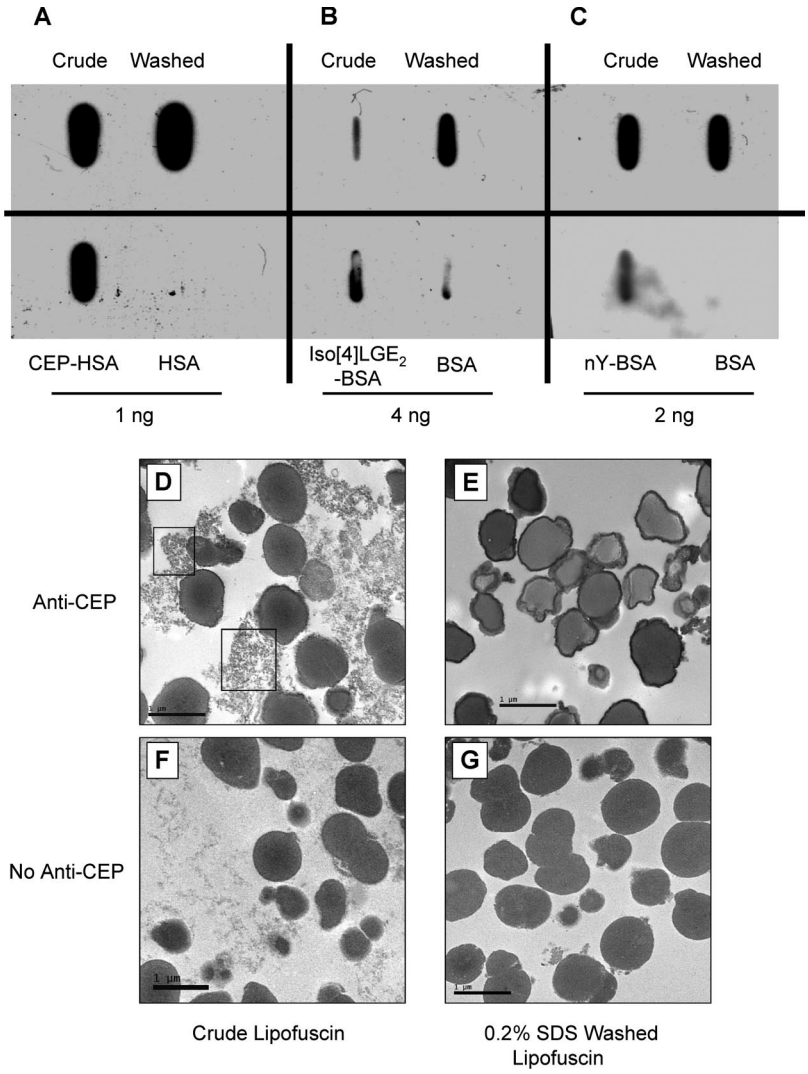

FIG. 2. **Oxidative modifications in lipofuscin.** Slot blot Western analyses of crude and SDS-washed lipofuscin (~4×10^7 granules applied per blot) are shown for immunoreactivity to CEP adducts (*A*), iso[4]LGE_2 adducts (*B*), and nitrotyrosine (*nY*) (*C*). Controls include CEP-modified and unmodified human serum albumin (*HSA*), iso[4]LGE_2-modified and unmodified BSA, and nitrotyrosine-modified and unmodified BSA with the applied amounts indicated. Separate slot blots of crude lipofuscin exhibited no immunoreactivity when probed with secondary antibody alone. Low level immunoreactivity for CEP and iso[4]LGE_2 adducts in unmodified albumin is commonly observed and likely reflects *in vivo* modification of this fatty acid-binding protein. Analyses by TEM of crude (*D*) and SDS-washed (*E*) lipofuscin for CEP immunoreactivity with anti-CEP primary antibody, horseradish peroxidase-conjugated secondary antibody, and DAB detection are shown. TEM control analyses with secondary antibody and DAB but no primary antibody are shown for crude lipofuscin (*F*) and SDS-washed granules (*G*). Intense CEP immunoreactivity is apparent on the granule surface (*D* and *E*) and in regions of the extragranular debris (*boxed* in *D*).

the presence of CEP immunoreactivity (supplemental Fig. 3). Immuno-TEM analyses also demonstrated CEP immunoreactivity on the surface of the lipofuscin granules in both purified and crude preparations and in the extragranular debris (Fig. 2, *D* and *E*). Control experiments lacking primary antibody showed no immunoreactivity in either crude or purified lipofuscin preparations by immuno-TEM (Fig. 2, *F* and *G*) or by Western slot blot (not shown).

Quantification of RPE Lipofuscin Protein by Amino Acid Analysis—Given the different results obtained from proteomics analysis of crude and purified lipofuscin preparations, amino acid analysis was used to quantify protein on a dry weight basis in crude, proteinase K-treated, and SDS-washed lipofuscin. These replicate amino acid analyses showed that the crude lipofuscin contained about 5 times more protein than the purified granules that contained ~2% (w/w) protein (Table I). No significant difference was detected in the total amount of amino acids in proteinase K-treated and SDS-washed lipofuscin granules. However, differences in amino acid composition were apparent for preparations from different donor groups (supplemental Tables 3 and 4).

TABLE I

Summary of amino acid content of lipofuscin preparations

Phenylthiocarbamyl amino acid analysis was performed on the above lipofuscin preparations as described under "Experimental Procedures." Preparation 1, 65 donors, mean age 70 years; preparation 3, 41 donors, mean age 76 years. The amino acid analysis data supporting this summary are presented in supplemental Tables 4 and 5.

Preparation	Number of analyses	Percent amino acid (w/w)	Average percent amino acid (w/w)
Crude preparation 1	3	9.2	10.8
Crude preparation 3	3	12.4	
Proteinase K-treated preparation 1	3	1.9	2.2
SDS-washed preparation 3	3	2.5	

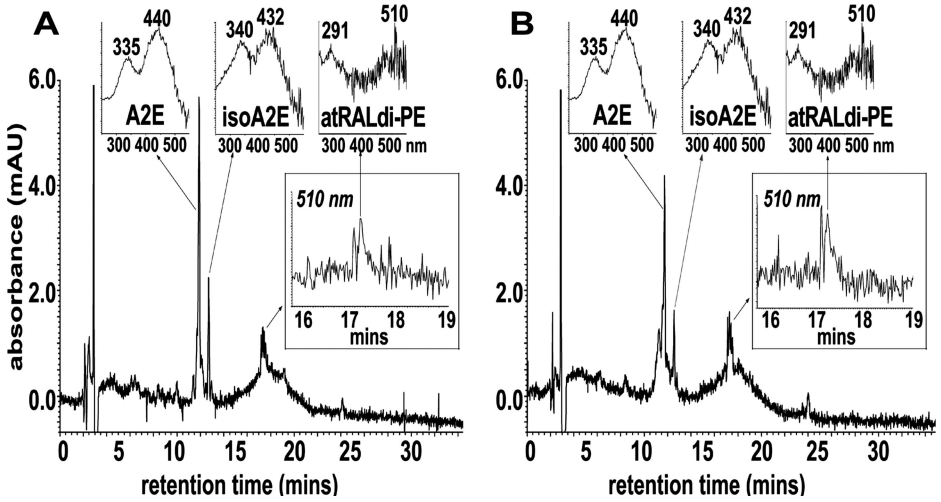

FIG. 3. **Quantitation of bisretinoid pigments in RPE lipofuscin.** Reverse phase HPLC analysis of chloroform/methanol extracts of equal amounts (~5.5 × 10^7 granules) of crude lipofuscin (*A*) and SDS-washed lipofuscin granules (*B*). Chromatograms were obtained with 430-nm monitoring. *Top insets*, UV-visible spectra of A2E, isoA2E, and all-*trans*-retinal dimer-phosphatidylethanolamine (*atRALdi-PE*) in acetonitrile/water. *Lower right insets*, monitoring at 510 nm, the detection wavelength favoring all-*trans*-retinal dimer-phosphatidylethanolamine, with the chromatogram expanded between retention times 15.5 and 19 min. The total amount of A2E and isoA2E recovered was ~380 pmol from the crude lipofuscin and ~328 pmol from the purified granules; the total amount of all-*trans*-retinal dimer-phosphatidylethanolamine was ~201 and ~187 pmol, respectively. These values are within experimental error of being equal given the 15.1% relative S.D. of the mean granule count. *mAU*, milliabsorbance units.

Quantification of Bisretinoids in RPE Lipofuscin—Equal amounts (by granule count) of crude or SDS-washed lipofuscin were extracted with chloroform and methanol and analyzed by reverse phase HPLC for A2E, isoA2E, and all-*trans*-retinal dimer-phosphatidylethanolamine (Fig. 3). No significant difference was detected in the total amount of these bisretinoids recovered from the crude and washed lipofuscin preparations, consistent with localization of the fluorophores to the granules.

Phototoxicity of RPE Lipofuscin—Equal amounts (by granule count) of crude or SDS-washed lipofuscin were fed to cultured ARPE-19 cells with or without exposure to intense blue light for 48 h, and the cells were assayed for viability. As expected from previous studies, there was no significant decrease in the viability of cells lacking lipofuscin and exposed to blue light or of cells fed lipofuscin but maintained in the dark (Fig. 4). However, ARPE-19 cells that were fed either with crude, membrane-bound lipofuscin granules or purified, membrane-free granules and exposed to blue light exhibited ~50% reduction in viability (Fig. 4). This result supports localization of the phototoxic activity of lipofuscin to the purified granules.

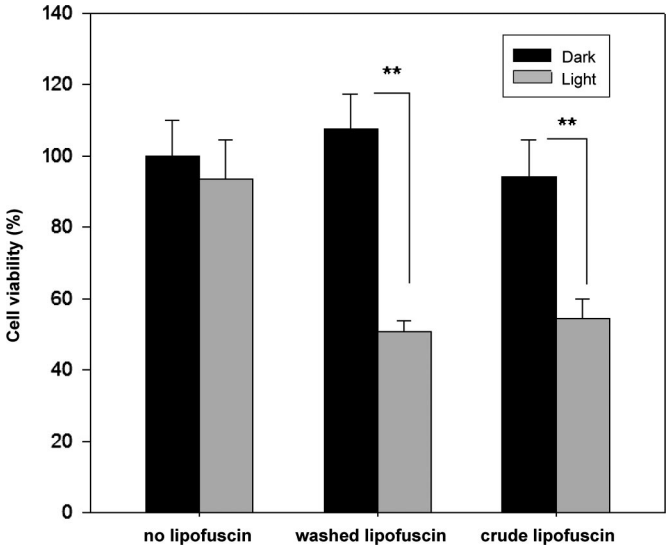

FIG. 4. **Phototoxicity of lipofuscin.** Cultured ARPE-19 cells were assayed for viability after treatment with equal amounts (300 granules/cell) of SDS-washed lipofuscin or crude lipofuscin with or without exposure to intense blue light for 48 h. Viability was calculated with reference to control cells not fed lipofuscin and maintained in the dark (set to 100%). Debris-free lipofuscin reduced ARPE-19 cell viability by the same amount as crude lipofuscin, namely ~50%. **, $p < 0.0001$, one-way analysis of variance. $n = 6$ except for dark-treated washed lipofuscin where $n = 3$. *Error bars* indicate standard error of the mean.

DISCUSSION

Toward a better understanding of RPE lipofuscin composition and pathogenesis, we purified RPE lipofuscin granules by treatment with proteinase K or SDS and demonstrated their purity by light, confocal, and transmission electron microscopy. Morphometric analyses showed little difference in the diameter, circularity, and roundness of the granules from purified or crude preparations with the average diameter (~0.75 ± 0.22 μm) agreeing well with the 0.7 μm value reported from atomic force microscopy (30). Removal of the extragranular debris was accompanied by loss of granule-associated membranes, but this was accomplished without reduction in the mass (size) of the fluorescent granules. Our proteomics analyses showed that purified lipofuscin granules

contain remarkably little protein (~2% (w/w) based on amino acid analysis), and no protein was identifiable by conventional mass spectrometric methods. Consistent with a lack of significant amounts of protein, the purified granules exhibited no colloidal Coomassie Blue detectable bands in SDS-PAGE or immunoreactive components following SDS-PAGE. However, Western slot blot and immuno-TEM analyses revealed the presence of oxidative protein modifications in the purified lipofuscin granules. In contrast, crude lipofuscin preparations were found to contain ~5 times more protein (w/w) and many identifiable proteins. We then evaluated the bioactivity of the purified granules and found no significant differences in phototoxicity or bisretinoid content between crude lipofuscin granules and the membrane-free, purified granules. These analyses confirm that the bioactivity of lipofuscin is associated with the granules (1, 2, 4) and, in contrast to previous reports, downplay a potential role for lipofuscin protein in pathological mechanisms.

Although our results indicate that lipofuscin granules contain only a small amount of protein, multiple reports of protein compositions for human RPE lipofuscin have contributed to a long standing misconception that lipofuscin contains substantial protein (6, 7). The resulting confusion highlights the importance of sample preparation for proteomics studies. Although we detected no proteins in purified granules, we identified 186 proteins in crude lipofuscin, including lysosomal membrane protein 2. Of the proteins identified by others in RPE lipofuscin, over 40% were detected in our crude lipofuscin preparations, strongly suggesting that the previous reports reflect the protein composition of extragranular contaminants and not of lipofuscin granules. About 20% of the proteins recently identified in RPE melanosomes (8) and ~33% of the proteins reported in RPE melanolipofuscin (9) were also identified in our analyses of crude lipofuscin. Notably extensive extragranular contamination is apparent in the published TEM images from these studies (6, 8, 9). RPE melanolipofuscin is another poorly defined fluorescent, granular waste material isolated by sucrose density gradient centrifugation with lipofuscin. The most common protein identified among the current study and the four other published proteomics reports concerning lipofuscin and melanolipofuscin was cathepsin D; all the common proteins are summarized in supplemental Table 5. A small fraction (<10%) of the proteins we detected in crude lipofuscin included visual cycle proteins from the RPE such as RPE65, cellular retinaldehyde-binding protein, and 11-*cis*-retinol dehydrogenase and photoreceptor proteins such as rhodopsin, cyclic GMP phosphodiesterase, and guanylyl cyclase among others. Little apparent similarity is evident between the protein composition we obtained for crude lipofuscin and that reported for phagosomes (31) and lysosomes (32) from non-RPE sources.

Oxidative stress has been associated with a host of age-related pathologies and long been suspected of contributing to lipofuscinogenesis. Our observations of CEP and iso[4]LGE_2 in purified granules are consistent with early theories suggesting a role for lipoxidation in lipofuscin formation (33). CEP adducts of protein primary amino groups are generated from 4-hydroxy-7-oxohept-5-enoic acid, a fragment derived uniquely from DHA-containing lipids (27) that are abundant in photoreceptor outer segments. In AMD, CEP adducts are elevated in Bruch membrane (27), the extracellular matrix separating the RPE from the blood-bearing choroid; AMD plasma also contains elevated levels of CEP adducts and CEP autoantibodies (34). Furthermore CEP adducts stimulate neovascularization *in vivo* (35) and can induce a late stage AMD-like phenotype (geographic atrophy) in a mouse model of AMD (36). Iso[4]LGE_2, an extraordinarily reactive electrophile belonging to the isolevuglandin family, is generated by free radical-induced oxidation of arachidonyl phospholipids and produces an array of lysyl modifications, including cross-links (29, 37). Elevated iso[4]LGE_2 adducts have been detected in plasma from patients with atherosclerosis and end stage renal disease (38). In addition, isolevuglandins can form DNA-protein cross-links (39) and inhibit mitosis and microtubule assembly (40). We cannot exclude the possibility that CEP and iso[4]LGE_2 adducts form with primary amino groups in sugars and lipids, but it has not yet been determined whether our antibodies recognize such epitopes. We detected nitrotyrosine in crude lipofuscin and the purified granules that is formed from the reaction of free or protein-bound tyrosine with nitrogen oxide species such as free radical nitrogen dioxide and peroxynitrite. A number of inflammatory and neurodegenerative disorders have been associated with tyrosine nitration including Parkinson, Alzheimer, and Huntington diseases (41). Other oxidative protein modifications reported previously in RPE lipofuscin include malondialdehyde and hydroxynonenal adducts and advanced glycation end products (10). It remains to be determined whether these other modifications are actually associated with the granules because the analyses were performed with crude lipofuscin preparations (10). Such protein modifications have the potential to mask proteolytic cleavage sites, inactivate enzymes (42), and contribute to the accumulation of partially degraded, cross-linked proteinaceous material. The small amount of protein detectable in lipofuscin granules by amino acid analysis likely represents heavily modified peptides and proteins that are refractory to proteolysis and protein identification by mass spectrometry. Such modifications would include but not be limited to nitrotyrosine, CEP, and iso[4]LGE_2.

This is the only study to date that has shown that RPE lipofuscin granules contain minimal protein. This suggests that lipofuscin protein has minimal impact on lipofuscin pathogenesis. We have demonstrated unequivocally that the toxic components of lipofuscin are associated with the granules, which contain phototoxic bisretinoids and many different lipids (43). Whether lipofuscin in nonocular tissues shares these properties remains to be determined. Previous studies

have demonstrated that RPE lipofuscin is a potent generator of reactive oxygen species including superoxide anion, singlet oxygen, and lipid hydroperoxides that have the capacity to escape the lysosome and modify other cellular compartments such as nuclear DNA and the plasma membrane as demonstrated in cell culture (4, 44–48). Given the highly conjugated structures of lipids and retinoids and their susceptibility to attack by free radicals and singlet oxygen, lipofuscin granules must be considered a rich source of highly reactive oxidative fragmentation products. Such reactive fragments would modify nucleophilic amino acids like Lys, Cys, and His within the lysosome and likely contribute to enzyme inactivation elsewhere in the cell via adducts like CEP and iso[4]LGE_2 and protein cross-linking. A2E has been shown to fragment upon irradiation, and complement has been shown to be activated in serum overlying irradiated A2E-containing RPE cells, raising the possibility that reactive fragments may escape from RPE cells (24). For example, a fraction of the DHA oxidative cleavage fragment 4-hydroxy-7-oxohept-5-enoic acid may escape from the RPE and contribute to the elevated plasma levels of CEP adducts and CEP autoantibodies in AMD patients (34). Toward a pathogenic mechanism for RPE lipofuscin, we hypothesize that oxidative cleavage of lipid and retinoid precursors within the granule generates a plethora of reactive fragments, some of which diffuse through membranes and modify intracellular and extracellular components with physiological consequences. The extent and consequences of lipofuscin-induced polyunsaturated fatty acyl and retinoid oxidation and subsequent protein or DNA modification would depend upon the antioxidant defense capability of the RPE and neighboring tissues. This hypothesis does not rule out possible roles for other granule components in lipofuscin pathogenesis, and further investigation is required to decipher the molecular details of the mechanisms of lipofuscin bioactivity.

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